Neural Grafting in the Mammalian CNS
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Peripheral Nerve Transplantation Techniques to Study Axonal Regeneration From the CNS of Adult Mammals

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I. Introduction

The failure of axons to regrow long distances through the damaged central nervous system (CNS) may be responsible for many of the functional deficits that occur as a consequence of injuries to the adult mammalian CNS. By contrast, axons damaged in the peripheral nervous system (PNS) can successfully regenerate when they become associated with Schwann cells and other PNS elements (Aguayo et al., 1979). Recent peripheral nerve transplantation studies have added further support to the hypothesis first proposed by Cajal that the type of environment that surrounds the axon tip is an important determinant of its regenerative response (Ramón y Cajal, 1928).

Here we describe experimental techniques in which peripheral nerve grafts have been used to investigate the growth and regeneration of axons arising from CNS neurons. The application of peripheral nerve grafts to the study of axonal regeneration derives from the occurrence of the following changes in excised nerve segments: (a) the axons and myelin sheaths degenerate rapidly; (b) Schwann cells divide and remain aligned in columns (Büngner bands) arranged longitudinally along the nerve segment; (c) each column is surrounded by a continuous 'tube' of basal lamina; (d) 'denervated' Schwann cells secrete a number of different molecules, some of which

have effects on nerve fiber growth (Richardson and Ebendal, 1982; Lundborg et al., 1982; Longo et al., 1983); and (e) other non-neuronal components of the original nerve (fibroblasts, collagen matrix, etc.) remain enclosed within the excised PNS segment. Thus, the chain-like arrangement of Schwann cells in denervated Büngner bands could provide growth cones with a continuous pathway and also with a unique relay system for the supply of growth factors.

After the transplantation of nerve segments into the CNS, new axons invade the graft and become ensheathed by Schwann cells. Using light and electron microscopy as well as different anterograde and retrograde anatomical tracing methods (Aguayo et al., 1983) and electrophysiological techniques (Munz et al., 1983, 1984), it has been possible to document that many of these axons arise from neurons that reside within the brain and spinal cord of the host animals. Furthermore, the course, length and termination of the regenerated central axons have also been studied with these techniques (Aguayo et al., 1983).

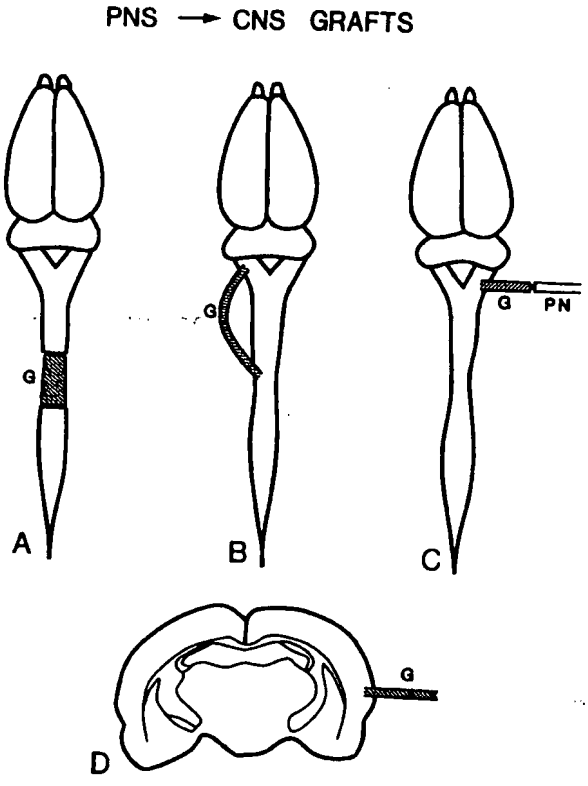

Figure 1. Schematic representation of four different ways in which peripheral nerve (PN) segments (G) can be transplanted into the CNS.

II. Types of Peripheral Nerve Transplantation Into the CNS

Peripheral nerve segments have been transplanted into the CNS in several different ways. Some of the most commonly used are: (a) joining the two stumps of the transected or hemisected spinal cord (Richardson et al., 1982) (Fig. 1A); (b) bridging two widely separated levels of the neuraxis (David and Aguayo, 1981) (Fig. 1B); (c) to guide central axons into peripheral tissues such as muscle or the distal stumps of peripheral nerves (Benfey and Aguayo, 1982; Richardson et al., 1984) (Fig. 1C); (d) serving as conduits for the unidirectional growth of CNS fibers (Richardson et al., 1984) (Fig. 1D); and (e) linking transplanted fetal neurons with adult host CNS (Aguayo et al., 1984) (Fig. 2).

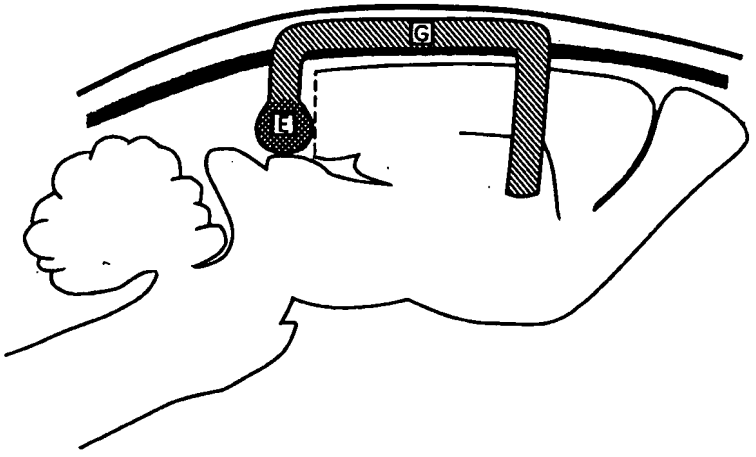

Figure 2. Schematic representation of transplanted fetal mesencephalon (E) connected through a peripheral nerve graft (G) to the head of the caudate-putamen. The mid-portion of the graft is placed extradurally.

III. Transplantation Techniques

The following is a description of some of the procedures we use for nerve grafting into the CNS of adult rats. We give a detailed account of the method to prepare PNS 'bridges' linking the medulla oblongata and the spinal cord (David and Aguayo, 1981), with additional instructions for the insertion of PNS grafts into other CNS regions.

III.1. PNS grafts as 'bridges' between the brain stem and the spinal cord

Equipment: (a) a stereoscopic dissecting microscope; (b) wet-field coagulator with bipolar forceps; (c) variable speed drill (Dremel).

Materials: microdissection instruments such as dissection forceps (#4 or #5); 5 in. rat toothed forceps; $3\frac{3}{4}$ in. straight scissors; $3\frac{1}{8}$ in. (straight) Vannas scissors; $4\frac{1}{2}$ in. scissors with 30 mm blades; 4 in. self retaining ratchet type tissue retractor; 5 in. microrongeur; $4\frac{5}{8}$ in. needle holder; 2-0, 5-0 and 10-0 sutures; micropipettes with a 100–150 μm tip.

Animals: 200–250 g, female rats.

Anesthesia: 7% chloral hydrate, injected intraperitoneally (0.6 ml/100 g body weight).

Obtaining segments of peripheral nerve: most segments are excised from the animal's own sciatic nerve. Allogenic or xenogenic grafts require immunosuppression of the host (Aguayo et al., 1979). (a) The skin of the posterior aspect of the hind leg is shaved and cleaned with 70% alcohol; (b) a scalpel incision is made from the sacrum to the popliteal fossa; (c) the gluteal muscles are cut and the hamstrings separated to expose the sciatic nerve; (d) for experiments requiring a graft with a length of approximately 2 cm, the common peroneal nerve branch is dissected and excised from the thigh and popliteal fossa – longer segments (3–4 cm) demand a more extensive exposure and dissection towards the sciatic notch and lower leg; (e) the removed segment is placed in cold Ringer's solution until the time of grafting; (f) in some experiments the transected nerve may be left in its place for 1–2 weeks to allow for the degeneration and removal of myelin and other cellular debris. However, it is our impression that such delayed procedure does not result in better graft innervation.

Preparation of the grafting sites: (a) after shaving and cleaning the occipital, cervical and upper thoracic regions, the skin and subcutaneous tissues are incised along the midline with a scalpel; (b) the neck muscles are separated along the midline and the occipital attachment of the paravertebral musculature is cut unilaterally to expose the atlanto-occipital ligament; (c) with microrongeurs a small portion of the occipital bone is removed, the bleeding is usually controlled with thrombin (Thrombostat, Parke Davis) soaked cotton swabs; (d) the grafting site in the spinal cord (usually in the lower cervical or upper thoracic spine) is then prepared by exposing and removing the spinous processes and laminae of two contiguous vertebrae; (e) to minimize graft innervation by sensory root axons the two dorsal root ganglia are avulsed.

Anchoring and inserting the graft: (a) after making a horizontal incision through the atlanto-occipital ligament with a pair of fine scissors, the epi-

neurium at the rostral end of the graft is sutured with 10-0 sutures to the lower edge of the cut ligament; (b) the nerve graft is placed along the paravertebral muscles with its caudal tip overlaying the selected spinal site of insertion; (c) the epineurium of the graft tip is similarly stitched to the dura mater; (d) to insert the nerve graft into the medulla oblongata and spinal cord the tips are trimmed and moistened – with the help of the glass micropipette the graft tips are inserted into the nervous tissues with a slow continuous movement aimed at reaching the desired structures; (e) the site of these structures is determined with stereotaxic coordinates – the depth of penetration can be marked on the micropipette; (f) tissues are closed in planes with sutures and animals are allowed to recover under a heat lamp.

Postoperative care: operated rats are housed in cages with smooth plastic bottoms to avoid injuring the insensitive limb with a wire mesh or other hard floorings.

III.2. Insertion of PNS grafts into other regions of the CNS

Cerebral hemispheres: after selecting the position of the targeted area (cortex, thalamus, striatum or other subcortical structures) with the help of a stereotaxic atlas of the rat brain (Pellegrino et al., 1979), a burr hole is drilled through the skull and enlarged with forceps or microrongeurs. The opening should be much larger than the diameter of the graft to facilitate the suturing of the epineurium to the dura mater. The dura is cut with the sharp tip of a 25-gauge needle and the tip of the graft is 'hinged' into the brain and inserted into the desired position with the micropipette or a thin stiff wire (Benfey and Aguayo, 1982).

Olfactory bulb: exposed surgically by drilling through the frontal bone, the bulb is delineated rostrally by the translucent olfactory mucosa and caudally by a venous sinus. The graft is anchored to the dura and inserted into the selected site through a small dural opening (Friedman and Aguayo, 1982, 1983, 1984).

III.3. PNS grafts linking transplanted fetal neurons with the adult host CNS

Nerve fibers arising from fetal neurons grafted into the brain (Björklund and Stenevi, 1984), cerebral ventricles (Freed et al., 1981) or anterior chamber of the eye (Olson et al., 1979) penetrate and branch extensively in the host tissues, but most of these axons only reach regions bordering the site of graft implantation. This makes it necessary for the neuronal transplant and host tissues to be placed in close proximity. The recent combination of fetal neu-

ronal and PNS grafts helps circumvent some of these limitations by allowing a wider separation of the source and targets of innervation.

Methods: here we describe briefly the bridging of an extracerebrally located fetal mesencephalic graft and the striatum of the adult host rat via a segment of peripheral nerve (Aguayo et al., 1984) (Fig. 2). It is anticipated that several different other combinations are feasible. In adult inbred rats: (a) the nigrostriatal dopamine pathway was damaged unilaterally by an intracerebral injection of 6-hydroxydopamine (8 μg free base in 4 μl, 0.2 mg/ml ascorbate saline). In addition, the superior cervical ganglion was extirpated bilaterally in all animals; (b) 6–8 weeks later a portion from the ventral mesencephalon of E16–17 rat embryos of the same strain as the host was placed intracranially over the superior colliculus. At the same time, a PNS 'bridge' measuring approximately 2 cm in length was obtained from the sciatic nerve of other rats of the same strain and placed along a bony groove drilled along the skull to the dural level. The epineurial sheath was slit at one end longitudinally and the nerve contents teased apart to form a 'pouch' over the fetal implant. The rostral end of the graft was inserted into the dorsal striatum either immediately or after several weeks; the latter measure is aimed at allowing the arrival of the nigral axons to the rostral end of the graft prior to its insertion into the basal ganglia.

This new experimental strategy provides a way for 'foreign' neuronal populations placed either intra- or extracerebrally to reach distant targets. Moreover, the enclosure of the fetal grafts within an epineurial 'pouch' helps direct fiber growth and minimizes its spread into neighboring regions.

IV. Technical Advantages

The peripheral nerve grafting techniques described above have several technical advantages as follows.

(1) Because most of the length of the grafts is in an extracranial or extravertebral location, retrograde markers can be applied to the grafts with little risk of spurious labeling of CNS neurons (David and Aguayo, 1981; Benfey and Aguayo, 1982).

(2) The length and accessibility of the grafts make them suitable for electrophysiological recordings of spontaneous and induced activity in the regenerated axons (Munz et al., 1983, 1984).

(3) It is possible to determine the source, number and pattern of distribution of CNS neurons that regenerate axons (Aguayo et al., 1983).

(4) It is possible to study the response of neurons in most regions of the CNS.

(5) The distance and rate of axon growth can be quantitated.

(6) The termination of the regenerated axons can be visualized by anterograde markers.

(7) The relationship between certain functional effects and axonal growth through the graft can be tested by blocking or transecting the grafts.

(8) Because most of the experimental animals have minimal motor and sensory loss and retain bowel and bladder control, their care and survival is greatly facilitated especially for long-term studies.

V. Present Limitations

In designing experiments it is important to be aware of the following limitations.

(1) The CNS neuronal populations recruited into growth represent but a small sample of the cells that either neighbor or project across the sites of injury and grafting.

(2) Most of the CNS neurons that regenerate axons into PNS grafts inserted into small incisions made in the spinal cord (David and Aguayo, 1981) or brain (Benfey and Aguayo, 1982) are situated within 1–6 mm of the graft tip. Grafts implanted into larger lesions that interrupt spinal long tracts have become innervated by a few cells located as far as 5 cm from the graft (Richardson et al., 1984), but we have never documented a regrowth from CNS cells situated beyond such distance.

(3) Not all nerve cells may be capable of growing axons into grafts. Possible examples are the small cells that normally lack such cellular processes (e.g. periglomerular and granule cells of the olfactory bulb) (Friedman and Aguayo, 1982, 1984) and perhaps also the cells of Purkinje in the cerebellum (Dooley and Aguayo, 1982).

(4) As a rule, PNS grafts are also invaded by axons of peripheral nerves situated nearby (meninges, blood vessels, cranial nerves, spinal roots).

(5) The target regions to which grafts are connected may also be a source of graft innervation. Thus, grafts often contain a two-directional innervation.

(6) Although central axons may regenerate several centimeters along PNS grafts, their re-entry into the CNS is limited to only a few millimeters; there is no evidence of an enhancement of axonal penetration into the CNS.

(7) Thus far, there is no proof that regenerating CNS axons make synaptic connections with neurons in the regions of the brain and spinal cord to which they are guided.

VI. Methods for the Study of Innervated Grafts

The innervation, course, terminal arborization, and functional properties of fibers extending along peripheral nerve grafts can be studied by various anatomical, histochemical, immunocytochemical and electrophysiological techniques.

The presence of axons in the grafts may be documented by light and electron microscopy of Epon-embedded material (Richardson et al., 1982; Aguayo et al., 1984), while their origin can be traced with different retrogradely transported markers, such as horseradish peroxidase (David and Aguayo, 1981; Richardson et al., 1982; Benfey and Aguayo, 1982) or fluorescent dyes (Fast Blue, True Blue and Nuclear Yellow) (David and Aguayo, 1982, 1984; Friedman and Aguayo, 1983, 1984). For this purpose the extracranial or extravertebral portion of the graft is exposed, cut and placed on a sheath of Parafilm and a gelfoam (Upjohn) pad soaked with the marker is applied to the tip of the graft. To avoid diffusion of the marker and spurious labeling of neurons, the remainder of the graft and surrounding regions are sealed with petroleum jelly. The origin, course and termination of the growing axons can be investigated by anterograde labeling with [^{3}H]amino acids (Richardson et al., 1982), histochemistry (Aguayo et al., 1984) or immunocytochemistry (Bray et al., this volume).

Finally, functional properties, such as spontaneous and evoked activity of the CNS neurons regenerating axons into the peripheral nerve graft, may be assessed electrophysiologically (Munz et al., 1983, 1984). In these experiments, the extracranial or extravertebral portion of the graft is exposed surgically and placed in a pool of mineral oil. The graft is then desheathed and teased to obtain fine nerve strands which are used for recording electrical activity.

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